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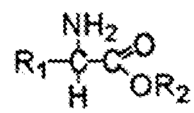
(57) Abstract



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Methods and compositions are provided for killing or inhibiting the growth of yeast or sporular microorganisms, in the presence of a peroxide and chloride or bromide, with a haloperoxidase and at least one antimicrobial activity enhancing agent. Suitable antimicrobial activity enhancing agents include certain alpha-amino acids, and are preferably compounds of formula (I) wherein R_1 is hydrogen, and unsubstituted, or hydroxy or amino substituted, straight or branched chain aklyl group having 1 to 6 carbon atoms, or an unsubstituted, or hydroxy or amino substituted arylalkyl group having from 7 to 12 carbon atoms, and R_2 is hydrogen or straight or branched chain alkyl group having from 1 to 6 carbon atoms.

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FUNGICIDAL METHODS AND COMPOSITIONS FOR KILLING YEAST AND SPORULAR MICROORGANISMS

Field of the Invention

This application is a continuation-in-part of U.S. application Serial No. 07/660,994 filed February 21, 1991.

The present invention relates to methods and compositions for the killing of yeasts and sporular forms of microbes. More particularly, the present invention relates to methods and compositions using a combination of an antimicrobial activity enhancing agent and haloperoxidase to enhance microbicidal properties of the system.

Background of the Invention

As disclosed in PCT application Publication Number WO 92/14484, haloperoxidases, such as myeloperoxidase and eosinophil peroxidase, may be used to selectively bind to and, in the presence of peroxide and halide, inhibit the growth of target microorganisms without eliminating desirable microorganisms or significantly damaging other components of the medium, such as host cells and normal flora, in the target microorganism's environment. Myeloperoxidase and eosinophil peroxidase have previously been known to exhibit microorganism killing activity in natural systems when presented with an appropriate halide cofactor (X^-) and hydrogen peroxide as substrate (Klebanoff, 1968, *J. Bacteriol.* 95:2131-2138). However, the selective nature of haloperoxidase binding and the utility of these systems for therapeutic, research and industrial applications has only recently been recognized. Due to the newly discovered selective binding properties of haloperoxidases, when a target microorganism, such as a pathogenic microorganism, has a binding capacity for haloperoxidase greater than that of a desired microorganism, such as members of the normal flora, the target microorganism selectively binds the haloperoxidase with little

or no binding of the haloperoxidase by the desired microorganism. In the presence of peroxide and halide, the target bound haloperoxidase catalyzes halide oxidation and facilitates the disproportionation of peroxide to singlet molecular oxygen ($^1O_2$) at the surface of the target microorganism, resulting in selective killing of the target microorganism with a minimum of collateral damage to the desired microorganism or physiological medium. Thus, as disclosed in PCT application Publication Number WO 92/14484, myeloperoxidase and eosinophil peroxidase can be employed as antiseptics in the therapeutic or prophylactic treatment of human or animal subjects to selectively bind to and kill pathogenic microorganisms with a minimum of collateral damage to host cells and normal flora of the host.

The system may also be employed in disinfecting or sterilizing formulations for inhibiting the growth of target microorganisms *in vitro*, particularly in applications where biomedical devices, such as bandages, surgical instruments, suturing devices, catheters, dental appliances, contact lenses and the like, are antiseptically treated to inhibit the growth of target microorganisms without damage to host cells of a subject when the biomedical device is subsequently utilized *in vivo*. While the haloperoxidase antiseptic system disclosed in PCT application Publication Number WO 92/14484 has been found to be highly effectively in the treatment of pathogenic microbes, yeast and some spore forming microorganisms remain relatively immune to haloperoxidase antimicrobial activity.

The spore stage of the microbial life cycle is characterized by metabolic dormancy and resistance to environmental factors that would destroy the microbe in its vegetative stage. The earliest phase of spore germination is characterized by swelling and a shift from dormancy to active metabolism. Vegetative growth, e.g., sprouting, and ultimately reproduction follows.

Germination of bacterial endospores and fungal spores is associated with increased metabolism and decreased resistance to heat and chemical reactants. For germination to occur, the spore must sense that the environment is adequate to support vegetation and reproduction. The amino acid *l*-alanine is reported to stimulate bacterial spore germination (Hills, 1950, *J Gen Microbiol* **4**:38; Halvorson and Church, 1957, *Bacteriol Rev* **21**:112). *L*-alanine and *l*-proline have also been reported to initiate fungal spore germination (Yanagita, 1957, *Arch Mikrobiol* **26**:329).

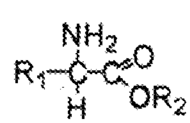
Simple α-amino acids, such as glycine and *l*-alanine, occupy a central position in metabolism. Transamination or deamination of α-amino acids yields the glycogenic or ketogenic carbohydrates and the nitrogen needed for metabolism and growth. For

example, transamination or deamination of *l*-alanine yields pyruvate which is the end product of glycolytic metabolism (Embden-Meyerhof-Parnas Pathway). Oxidation of pyruvate by pyruvate dehydrogenase complex yields acetyl-CoA, NADH, H^+, and CO_2. Acetyl-CoA is the initiator substrate for the tricarboxylic acid cycle (Kreb's Cycle) which in turns feeds the mitochondrial electron transport chain. Acetyl-CoA is also the ultimate carbon source for fatty acid synthesis as well as for sterol synthesis. Simple α-amino acids can provide the nitrogen, CO_2, glycogenic and/or ketogenic equivalents required for germination and the metabolic activity that follows.

Zgliczxnski et al. (1968, *European J. Biochem* **4**:540-547) reported that myeloperoxidase catalyzes the chloride-dependent oxidation of amino acids by hydrogen peroxide to yield ammonia, carbon dioxide and an aldehyde corresponding to the decarboxylated, deaminated amino acid, and Strauss et al. (1970, *J. Reticuloendothel Soc* 7:754-761) postulated that the aldehydes so produced might serve as microbicidal agents. However, Paul et al. (1970, *Infect Immun* **2**:414-418) reported that adding the α-amino acids glycine and *l*-alanine to a myeloperoxidase-hydrogen peroxide-chloride test system actually inhibited killing of *Escherichia coli*. Furthermore, Klebanoff (1975, *Semin Hemat* **12**:117-142) reported that 100 mM acetaldehyde was required for bactericidal action. Contrary to these published data, it has now been surprisingly discovered that the microbicidal action of haloperoxidases against yeast and sporular forms of microbes may be significantly enhanced by treating the microorganisms with haloperoxidase in combination with certain α-amino acids which serve as an antimicrobial activity enhancing agent.

Summary of the Invention

In accordance with the present invention, methods and compositions are provided for killing or inhibiting the growth of yeast or sporular microorganisms comprising contacting the microorganisms, in the presence of a peroxide and chloride or bromide, with a haloperoxidase and at least one antimicrobial activity enhancing agent. Suitable antimicrobial activity enhancing agents include certain α-amino acids, and are preferably compounds of the formula:



wherein R_1 is hydrogen, an unsubstituted, or hydroxy or amino substituted, straight or branched chain alkyl group having from 1 to 6 carbon atoms, or an unsubstituted, or hydroxy or amino substituted arylalky group having from 7 to 12 carbon atoms, and R_2 is hydrogen or a straight or branched chain alkyl group having from 1 to 6 carbon atoms. In one embodiment, the methods and compositions of the invention may be used to kill yeast and sporular microbes *in vitro*, to disinfect or sterilize medical products or materials. In other embodiments, the methods and compositions can be employed in the antifungal and antiyeast treatment of human or animal subjects without eliminating desirable microorganisms or significantly damaging host cells. It has been discovered that the antiyeast and antifungal spore activities of haloperoxidases are significantly enhanced in the presence of certain α-amino acids. In the further presence of peroxide and halide, the target bound haloperoxidase catalyzes halide oxidation and facilitates the disproportionation of peroxide to singlet molecular oxygen at the surface of the spore forming microorganism, resulting in killing of the target microorganism. Although it is likely that haloperoxidase activity will catalyze the deamination, decarboxylation of a portion of the added α-amino acids to yield aldehydes, it is unlikely that such aldehydes significantly contribute to microbicidal action at such low concentrations. It is likely that these α-amino acids exert a mild concentration-dependent competitive inhibition of microbicidal action by consuming a portion of the haloperoxidase generated hypochlorous acid and singlet molecular oxygen. However, the stimulating effect of these α-amino acids on yeast budding, germination of sporulated microbes, and possibly acceleration of metabolism of vegetative microbes appears to labilize the microbes so treated to the actions of haloperoxidases and thus greatly enhance microbicidal action.

The significantly enhanced haloperoxidase antiyeast and antispore activities make the methods and compositions of the invention highly useful in the therapeutic or prophylactic antiseptic treatment of human or animal subjects and in *in vitro* applications for disinfecton and sterilization of vegetative microbes, yeasts, and bacterial and fungal spores.

Suitable haloperoxidases for use in the methods and compositions of the invention include eosinophil peroxidase (EPO) and myeloperoxidase (MPO). Representative antimicrobial activity enhancing agents of the invention include α-amino acids selected from the group consisting of glycine and the *l*- or *d*-enantiomers of alanine, valine, leucine, isoleucine, serine, threonine, lysine, phenylalanine, tyrosine, and the alkyl esters thereof.

Detailed Description of the Preferred Embodiment

The present invention is broadly directed to methods and compositions for the killing or inhibition of yeast and sporulated microorganisms using a haloperoxidase and an antimicrobial activity enhancing agent which labilizes the yeast and spore forms of the microorganism to haloperoxidase microbicidal activity. In the practice of the invention, yeast and spore forms of microorganisms are killed or inhibited by contacting the microorganisms with amounts of a haloperoxidase and an antimicrobial activity enhancing agent, i.e., certain α-amino acids, which are effective in the presence of a peroxide and bromide or chloride, to inhibit the growth of or kill the microorganisms.

In one particularly preferred embodiment, the methods and compositions of the invention are used as antiseptic agents exhibiting enhanced haloperoxidase antispore and antiyeast activity against a broad range of pathogenic microorganisms including bacteria and fungi. For use in contact with host tissue, the antiseptic systems are based on the use of dioxygenating haloperoxidase enzymes which exhibit selective affinity for pathogenic microorganisms. As such, high potency microbicidal action can be directed to the target microorganisms without associated host tissue destruction or disruption of normal flora; i.e., the antiseptic action is selective and confined to the target microorganism.

When properly formulated, haloperoxidase-enhancer preparations can be employed to disinfect and even sterilize materials and devices. High potency haloperoxidase-enhancer formulations can serve as *in vitro* disinfecting or sterilizing preparations. By limiting the time period of hydrogen peroxide availability, haloperoxidase-enhancer formulations can be made sufficiently potent to insure disinfection and even sterilization of a material or device before contact with host tissue. Any potential toxicity to normal flora and host tissue associated with the use of these high potency formulations will cease when peroxide is depleted, and as such, the formulation-treated material or device can be brought in contact with host tissue without additional washing to detoxification.

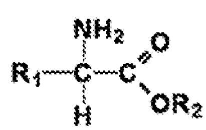
Representative compositions of the invention comprise (1) eosinophil peroxidase (EPO) and/or myeloperoxidase (MPO), (2) hydrogen peroxide (H_2O_2) or equivalent peroxide, or an oxidase for the generation of H_2O_2, (3) a substrate for the oxidase, and (4) an antimicrobial activity enhancing agent, such as glycine or *l*-alanine.

In one presently preferred embodiment, the invention provides methods and compositions for inhibiting the growth of yeast and sporular microorganisms *in vitro*, particularly in applications where biomedical devices, such as bandages, surgical

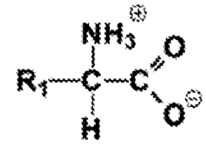
instruments, suturing devices, catheters, dental appliances, contact lenses and the like, require disinfection or sterilization and where the device is to be subsequently contacted with host tissue. The methods and compositions of the invention may also be used to treat or prevent infections by yeast or spore forming microorganisms *in vivo*.

Haloperoxidases useful in the present invention are defined as halide:hydrogen peroxide oxidoreductases (e.g., EC No. 1.11.1.7 and EC No. 1.11.1.10 under the International Union of Biochemistry) for which halide, i.e., chloride or bromide, is the electron donor or reductant and peroxide is the electron receiver or oxidant. Any haloperoxidase which catalyzes the halide dependent generation of singlet molecular oxygen from hydrogen peroxide and which exhibits selective binding to target microorganisms may be used in the present invention. Presently particularly preferred haloperoxidases, as demonstrated herein, include eosinophil peroxidase (EPO), myeloperoxidase (MPO) and combinations thereof. Inclusion of an antimicrobial enhancing agent, as described in detail herein, greatly increases the microbicidal capacity of the oxidase-haloperoxidase system against yeast and sporular microorganisms since it labilizes these forms to the microbicidal action of the haloperoxidase system.

Antimicrobial activity enhancing agents of the invention are agents that enhance the antimicrobial activity of the haloperoxidase antimicrobial system against yeast and sporular microorganisms, used at concentrations that do not produce adverse effects on the haloperoxidase activity of the system or undesirable effects in the environment of use of the methods and compositions. Presently preferred activity enhancing agents of the invention include α-amino acid compounds of the formula:



wherein R_1 is hydrogen, a straight or branched chain alkyl group having from 1 to 6 carbon atoms, or an unsubstituted or hydroxy or amino substituted straight or branched chain arylalky group having from 7 to 12 carbon atoms, and R_2 is hydrogen or a straight or branched chain alkyl group having from 1 to 6 carbon atoms. As used herein, amino acids may be in their acid form, as shown above, or may be in their zwitterionic form represented by the formula:



wherein R_1 and R_2 having the meanings set forth above, and may be in either *l*- or *d*-enantiomeric configurations. Representative alkyl R_1 groups include, for example, methyl, hydroxymethyl, isopropyl, 2-isobutyl, 1-isobutyl, hydroxy ethyl and amino butyl groups. Representative arylalkyl R_1 groups include, for example, tolyl and hydroxytolyl groups. Presently particularly preferred alkyl R_2 groups include methyl and ethyl groups. Representative antimicrobial activity enhancing agents of the invention include α-amino acids selected from the group consisting of glycine and the *l*- or *d*-enantiomers of alanine, valine, leucine, isoleucine, serine, threonine, lysine, phenylalanine, tyrosine and the alkyl esters thereof. The presently most preferred antimicrobial activity enhancing agents are glycine and *l*-alanine.

The nature and thickness of the spore wall affords protection against the lethal action of singlet molecular oxygen and hypochlorous acid. With respect to fungal spores, α-amino acid induced spore germination yields vegetative forms that are more susceptible to oxidants. In addition, it has been found that the antimicrobial activity enhancing agents of the invention also increase oxidase-haloperoxidase killing of yeast vegetative forms, including *Candida albicans* (see **Table 1**, below). This phenomenon may be related to the α-amino acid-dependent acceleration of yeast growth and budding, and the increased susceptibility of such metabolically active forms to haloperoxidase killing. One alternative possibility is that α-amino acids, or metabolic products thereof, act as a substrate for a fungal oxidase capable of generating H_2O_2. Another alternative possibility is that the aldehyde products of haloperoxidase-mediated α-amino acid deamination-decarboxylation might induce germination and budding, or otherwise effect some vital process.

Since the antiseptic activity of the haloperoxidase compositions of the invention involves the reaction of peroxide and bromide or chloride to form hypohalite, and the reaction of peroxide and hypohalite to form singlet molecular oxygen, the activity of the compositions of the invention is dependent upon the presence, at the site of antimicrobial activity, of a suitable peroxide and halide. In some situations, peroxide (e.g., hydrogen peroxide) may be present at the site of antimicrobial activity due, for example, to the activity of naturally occurring flora, and sufficient amounts of chloride may be present in the physiological milieu to act as a cofactor in the conversion reaction. In these situations, no additional peroxide or

halide need be administered or included in the compositions of the invention. In other situations, it may be necessary to additionally provide hydrogen peroxide and/or halide at the site of microbial treatment. Accordingly, the compositions of the invention may additionally comprise, if desired, a peroxide or agent capable of producing peroxide *in vivo* or *in vitro* and a halide.

Peroxides useful in the methods and compositions of the invention include hydrogen peroxide, alkyl hydroperoxides of the formula:

$$R\text{-}OOH$$

wherein R is a hydrogen or a short chain alkyl group having from 1 to 3 carbon atoms, and inorganic peroxides, such as boroperoxide or ureaperoxide. The oxidant activity of the organic peroxides generally decreases with increasing R chain length, as follows:

$$R{=}H >> CH_3 > CH_3CH_2 > CH_3(CH_2)_2$$

The presently preferred peroxide for use in the compositions of the invention is hydrogen peroxide. Hydrogen peroxide may also be made available at the site of the antimicrobial activity by including in the composition an agent capable of producing hydrogen peroxide *in vivo* or *in vitro*. Particularly useful agents for this purpose include, for example, oxidases, such as cholesterol oxidase, glucose oxidase and galactose oxidase.

When hydrogen peroxide is directly included in compositions of the invention for *in vivo* applications, the amounts employed are preferably designed to provide maximum disinfecting activity. Damage to host cells and tissue and to normal flora is avoided by avoiding direct contact during the period of high H_2O_2 exposure. Accordingly, when included in liquid formulations, the compositions of the invention may comprise from about 1 nmol to about 10 μmol of hydrogen peroxide per ml of liquid composition, more preferably from about 5 nmol to about 5 μmol of hydrogen peroxide per ml of liquid composition, and most preferably from about 10 nmol to about 1 μmol of hydrogen peroxide per ml of liquid composition. Agents capable of producing hydrogen peroxide *in vivo*, e.g., peroxide producing oxidases, are particularly useful for dynamic control of the amounts of hydrogen peroxide present at the site of antimicrobial activity. Such agents maximize antimicrobial activity of the composition by providing and maintaining a steady, low level concentration of H_2O_2. Accordingly, the amount of such agents to be employed will be highly dependent on the nature of the agent and the effect desired, but will preferably be capable of producing a steady state level of from about 1 pmol to about 1 μmol of hydrogen peroxide per ml of liquid per minute, depending on the type and concentration of

halide available at the site of antimicrobial activity. When the formulation is to be used as a disinfectant-sterilizing solution, the oxidase and its substrate can be adjusted to provide relatively high steady-state concentrations of H_2O_2 lasting for the required sterilization period. The disinfection-sterilizing action is terminated with exhaustion of the oxidase substrate or relative to the rate of oxidase degradation.

For antifungal purposes, the use of cholesterol oxidase, e.g., from *Nocardia erythropolis*, as a H_2O_2 producing oxidase is presently particularly preferred. Unlike prokaryotic bacteria, fungi synthesize sterols. In fact, the antifungal activity of amphotericin B is at least in part dependent on binding to fungal membrane steroids, e.g., ergosterol. Ergosterol is the predominant sterol constituent of most fungi, but other sterols are present (Weete, 1973, *Phytochemistry* **12**:1843). Cholesterol oxidase from *Nocardia erythropolis* selectively oxidizes Δ^5-3β-ols and 5α-3β-ols to the resulting ketones (Smith and Brooks, 1974, *J Chromatography* **101**:373); e.g.,

$$\text{cholesterol} + O_2 \xrightarrow{\textit{cholesterol oxidase}} \text{cholestenone} + H_2O_2$$

$$\text{ergosterol} + O_2 \xrightarrow{\textit{cholesterol oxidase}} \text{ergostenone} + H_2O_2$$

This *Nocardia* oxidase is relatively heat stable and retains catalytic activity at 50°C. It is active over a pH range of 4 to 9 with a maximum activity at pH 7. It has a Michaelis constant (Km) of 1.4×10^{-5} mol/liter (Richmond, 1973, *Clin Chem.* **19**:1350).

Haloperoxidases are fungicidal when presented with H_2O_2 or coupled to a H_2O_2-generating oxidase. However, with cholesterol oxidase as the oxidase, oxidase-haloperoxidase fungal killing is greater than expected from the generation of H_2O_2 alone. This cholesterol oxidase-dependent increase in fungicidal action may in part be related to disruption of fungal membrane integrity resulting from oxidase depletion of fungal steroids. Fungi might also synthesize an endogenous H_2O_2-generating sterol oxidase.

Suitable halides for use in the methods and compositions of the invention may be bromide or chloride. The use, selection, and amount of halide employed in a particular application will depend upon various factors, such as the haloperoxidase used in the antiseptic composition, the desired therapeutic effect, the availability of peroxide and other factors. When the haloperoxidase is myeloperoxidase, the halide may be bromide or chloride. Since chloride is present in most physiological media at levels sufficient to be nonlimiting as the halide cofactor, an external source of chloride is generally not required. When an external source of chloride is desired, the amount of chloride employed will preferably fall in the range of about 10 μmol chloride to about 150 μmol chloride per ml of solution to approximate physiological conditions.

When the haloperoxidase is eosinophil peroxidase, chloride is relatively ineffective as a cofactor, and accordingly, the preferred halide is bromide. When included in liquid compositions, the compositions of the invention may comprise from about 1 nmol bromide to about 20 μmol bromide per ml of liquid composition, more preferably from about 10 nmol bromide to about 10 μmol bromide per ml of liquid composition, and most preferably from about 100 nmol bromide to about 1 μmol bromide per ml of liquid composition.

The ratio of halide to peroxide is an important consideration in formulating an effective microbicidal environment. Accordingly, in addition to ensuring effective levels of halide and peroxide at the situs of microbial attack, as described above, it is preferable to practice the methods of the invention at halide:peroxide ratios that provide optimal microbicidal activity. For example, when the haloperoxidase is MPO and the halide is Cl^-, the ratio of Cl^- to peroxide is preferably maintained in the range of about 1 to about 40,000 in the environment of microbicidal activity, more preferably from about 50 to about 40,000 and most preferably from about 200 to about 40,000. When the halide is Br^-, the ratio of Br^- to peroxide is preferably maintained in the range of about 0.1 to about 4,000 in the environment of microbicidal activity, more preferably from about 0.5 to about 2,000 and most preferably from about 1 to about 1,000.

The methods and compositions of the invention can be used to inhibit the growth of a broad spectrum of sporular microorganisms, preferably with a minimum of damage to normal flora. As used herein, "sporular microorganisms" is intended to include spore forms of bacteria or fungi. Spore forming microorganisms are well known, and include, for example, bacteria such as *Bacillus* sps. and *Clostridium* sps., and fungi such as *Aspergillis* sps., *Fusarium* sps., *Trichophyton* sps. and the like.

As used herein, the term "normal flora" means bacteria which normally reside in or on body surfaces of a healthy host at symbiotic levels. Normal flora include, for example, the lactic acid family of bacteria in the mouth, intestine, or vagina of human subjects, e.g. *Streptococcus* (viridans) in the mouth, and *Lactobacillus sp.* (e.g., Tissier's bacillus and Doderlein's bacillus) in the intestines of breast-fed infants, external genitalia, anterior urethra and vagina. Microorganisms which constitute normal flora of a host are well known (e.g., see *Principles and Practice of Infectious Diseases*, supra, New York, pp. 34-36 and 161). It has been found that the haloperoxidases of the invention selectively bind to many pathogenic bacteria and fungi in preference over normal flora. In *in vivo* applications, the host is preferably treated with an amount of haloperoxidase which is ineffective to eliminate normal

flora from the host. In *in vitro* applications for disinfection-sterilization, sufficiently high concentrations of haloperoxidase can be employed to ensure complete killing of all vegetative and yeast forms. Under such conditions, damage to host tissue and normal flora is avoided by consumption of H_2O_2 or the H_2O_2-generating system prior to contact with the host tissue.

The compositions of the invention generally comprise amounts of a haloperoxidase and of an antimicrobial activity enhancing agent which are effective, in the presence of a peroxide and a halide to kill or inhibit the growth of yeast or sporular microorganisms. The compositions may be conveniently provided in a liquid carrier. Any liquid carrier may be generally used for this purpose, provided that the carrier does not significantly interfere with the selective binding capabilities of the haloperoxide or with enzyme activity. Alternatively, the compositions may be provided in solid form with activation on solubilization in liquid.

The compositions of the invention may additionally comprise peroxide or an agent capable of producing peroxide, such as an oxidase, as described in detail above. The oxidase-haloperoxidase system lends itself to construction as a binary formulation. One part of the binary comprises a solution containing the oxidase, the haloperoxidase and the antimicrobial activity enhancing substance, e.g., glycine or *l*-alanine. The second part of the binary comprises a substrate for the oxidase, e.g., cholesterol in the case of cholesterol oxidase or molecular oxygen, O_2. The substrate may be provided, for example, in the form of a solid wafer. For sterilization of an article, e.g., a contact lens, the cholesterol wafer is placed in a sterilization chamber along with the item to be sterilized. The cholesterol oxidase-haloperoxidase plus glycine or *l*-alanine solution is added to initiate sterilization. This composition may additionally comprise alcohol in order to facilitate cholesterol solubilization and utilization by the oxidase. This system will produce sustained microbicidal action as long as sufficient cholesterol is present to drive the reaction.

For *in vivo* applications, the antiseptic compositions can be administered in any effective pharmaceutically acceptable form to warm blooded animals, including human and animal subjects, e.g., in topical, lavage, oral or suppository dosage forms, as a topical, buccal, or nasal spray or in any other manner effective to deliver active haloperoxidase to a site of microorganism infection. The route of administration will preferably be designed to obtain direct contact of the antiseptic compositions with the infecting microorganisms.

For topical applications, the pharmaceutically acceptable carrier may take the form of liquids, creams, foams, lotions, or gels, and may additionally comprise organic

solvents, emulsifiers, gelling agents, moisturizers, stabilizers, surfactants, wetting agents, preservatives, time release agents, and minor amounts of humectants, sequestering agents, dyes, perfumes, and other components commonly employed in pharmaceutical compositions for topical administration.

Solid dosage forms for oral or topical administration include capsules, tablets, pills, suppositories, powders, and granules. In solid dosage forms, the compositions may be admixed with at least one inert diluent such as sucrose, lactose, or starch, and may additionally comprise lubricating agents, buffering agents, enteric coatings, and other components well known to those skilled in the art.

In another embodiment of the invention, the compositions of the invention may be specifically designed for *in vitro* applications, such as disinfecting or sterilization of medical devices, contact lenses and the like, particularly where the devices or lenses are intended to be used in contact with a patient or wearer. For applications of this type, the compositions may be conveniently provided in the form of a liquid or foam, and may be provided with emulsifiers, surfactants, buffering agents, wetting agents, preservatives, and other components commonly found in compositions of this type. Compositions of the invention may be impregnated into absorptive materials, such as sutures, bandages, and gauze, or coated onto the surface of solid phase materials, such as staples, zippers and catheters to deliver the compositions to a site for the prevention of microbial infection. Other delivery systems of this type will be readily apparent to those skilled in the art.

Actual amounts of haloperoxidase and antimicrobial activity enhancing agents in the compositions of the invention may be varied so as to obtain amounts of haloperoxidase and antimicrobial activity enhancing agents at the site of treatment effective to kill vegetative as well as yeast and sporular microorganisms. Accordingly, the selected amounts will depend on the nature and site for treatment, the desired response, the desired duration of microbicidal action and other factors. Generally, when the haloperoxidase is myeloperoxidase, liquid compositions of the invention will comprise at least 0.01 picomoles (pmol) of myeloperoxidase per ml of liquid composition, more preferably from about 0.1 pmol to about 500 pmol of myeloperoxidase per ml of liquid composition, and most preferably from about 0.5 pmol to about 50 pmol of myeloperoxidase per ml of liquid composition. Similar dosages of eosinophil peroxidase may be employed. Optionally, it may be desirable in some applications to include both eosinophil peroxidase and myeloperoxidase in the same composition. Liquid compositions of the invention will generally comprise at least 0.005 μmol/ml of antimicrobial activity enhancing agents, i.e., α-amino acids

such as glycine and alanine, and more preferably from 0.05 μmol/ml to 50 μmol/ml of such antimicrobial activity enhancing agent.

Other components, such as an oxidase for peroxide generation, substrate for the oxidase and halide may be included, as desired, as described in detail above. In addition, the components may be formulated in a single formulation, or may be separated into binary formulations for later mixing during use, as may be desired for a particular application. For single formulations, one required system component which is available at the application site, such as halide, oxidase, prosthetic group for the oxidase, reducing substrate for the oxidase, or molecular oxygen is preferably left out of the formulation to preclude premature reaction and exhaustion of system components.

As an illustrative example, a composition suitable for use as a contact lens solution may comprise from 1 to 20 pmol/ml of eosinophil peroxidase and/or myeloperoxidase, from 0.1 to 1 μmol/ml of glycine, from 0.01 to 10 units of glucose oxidase, and from 50 to 500 mEq/L of chloride with 0.1 to 1 mEq/L bromide. The above composition is combined with from 1 to 10 μmol/ml of glucose under anaerobic conditions and the complete preparation is kept anaerobic until used as a liquid disinfectant or sterilizing solution. Exposure to air, i.e., molecular oxygen, activates the disinfecting-sterilizing action of the formulation.

The foregoing may be better understood in connection with the following representative examples, which are presented for purposes of illustration and not by way of limitation.

EXAMPLES

Example 1

The Effect of *l*-Alanine on Oxidase-Haloperoxidase Killing of Bacterial, Yeast and Fungal Spores

The effect of *l*-alanine on oxidase-haloperoxidase killing of bacterial, yeast and fungal spores was determined as follows. Incubation media was prepared from 50 mM acetate buffer containing 0.1 unit (i.e., 4 μg) cholesterol oxidase from *Nocardia erythropolis* (prepared in accordance with the procedure of Richmond, W., "Preparation and Properties of a Cholesterol Oxidase from *Nocardia* sp. and Its Application to the Enzymatic Assay of Total Cholesterol in Serum," *Clin. Chem.* **19(12)**:1350-1356 (1973), 20 pmol (2.8 μg) porcine MPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1899201) or 20 pmol (1.5 μg) porcine EPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1929201), 100 mEq/L Cl^-, and 1 mEq/L Br^-. Incubation mixtures were prepared by inoculating the incubation media

with 1 x 10^7 cells of *Staph. aureus*, *Cand. albicans*, and *Asperg. fumigatus*. The pH of the incubation mixtures was adjusted to 7 with 50 mM MOPS buffer. Cholesterol in 8.5% ethanol was added to the incubation mixtures to a final concentration of 7 mM (7 µmol/ml). The final volume of the incubation mixtures was 1 ml. The mixtures were incubated for four hours at 22°C and the microbes were then plated (*S. aureus* was plated on trypticase soy agar; *C. albicans* and *A. fumigatus* were plated on Sabouraud's dextrose agar). After about 24 hours (about 72-96 hours for *A. fumigatus*), the colony forming units (CFU) were counted as a measure of the viability of the organisms. The results are shown in **Table 1**.

Table 1
Effect of *l*-Alanine on Cholesterol Oxidase-Haloperoxidase Microbicidal Action Against *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus fumigatus* Spores:

Organism	Cholesterol Oxidase	Haloperoxidase	CFU
Staph. aureus	None	None	19,400,000
Staph. aureus	0.1 Unit	None	29,000,000
Staph. aureus	0.1 Unit †	None	29,200,000
Staph. aureus	0.1 Unit	20 pmol MPO	0
Staph. aureus	0.1 Unit †	20 pmol MPO	0
Staph. aureus	0.1 Unit	20 pmol EPO	0
Staph. aureus	0.1 Unit †	20 pmol EPO	0
Cand. albicans	None	None	1,460,000
Cand. albicans	0.1 Unit	None	1,380,000
Cand. albicans	0.1 Unit †	None	1,580,000
Cand. albicans	0.1 Unit	20 pmol MPO	800,000
Cand. albicans	0.1 Unit †	20 pmol MPO	0
Cand. albicans	0.1 Unit	20 pmol EPO	680,000
Cand. albicans	0.1 Unit †	20 pmol EPO	0
Asperg. fumigatus	None	None	1,260,000
Asperg. fumigatus	0.1 Unit	None	1,020,000
Asperg. fumigatus	0.1 Unit †	None	880,000
Asperg. fumigatus	0.1 Unit	20 pmol MPO	550,000
Asperg. fumigatus	0.1 Unit †	20 pmol MPO	0
Asperg. fumigatus	0.1 Unit	20 pmol EPO	840,000
Asperg. fumigatus	0.1 Unit †	20 pmol EPO	0

† indicates that the 50 mM Acetate Buffer contained 1 mM *l*-alanine.

As shown in **Table 1**, cholesterol oxidase plus either MPO or EPO provides a potent microbicidal system. This combination killed 10^7 *Staphylococcus aureus* in the presence or absence of 1 mM *l*-alanine. However, inclusion of *l*-alanine in the cholesterol oxidase-haloperoxidase system was necessary for complete killing of both *Candida albicans* yeast forms and *Aspergillus fumigatus* spores.

Example 2

Effect of Potential Amino Acid Antimicrobial Activity Enhancing Agents on Haloperoxidase Microbicidal Action Against *Aspergillus fumigatus* Spores

The effect of various potential amino acids as enhancing agents for haloperoxidase microbicidal action was determined by following the procedure of Example 1, except that each test contained the quantity of glucose oxidase indicated in **Tables 2-8**, below (instead of cholesterol oxidase as in Example 1), in an incubation medium of 5.6 mM glucose in 50 mM sodium acetate buffer containing 100 mEq/L of chloride and 0.1 mEq/L of bromide at pH 6. The potential amino acid activators indicated in **Tables 2-8** below were added to the mixtures to a final concentration of 0, 5, 0.5 or 0.05 μmol/ml, and the incubation mixtures were innoculated with about 1 x 10^7 spores of *Aspergillus fumigatus*. The mixtures were incubated at ambient temperature for 90 minutes and then plated as described in Example 1. The plates were grown overnight at 35°C, and then for an additional two days. The colony forming units were counted as a measure of viability of the organisms.

The aliphatic amino acids glycine, *l*-alanine, *l*-valine, *l*-leucine and *l*-isoleucine were tested as described above. The results are shown in the following **Table 2**:

Table 2
Amino Acid Type and Concentration: Effect on Haloperoxidase Killing of *Aspergillus fumigatus* Spores

Haloperoxidase	Glucose Oxidase	(Amino Acid) µmol/ml (mM)	CFU (Aliphatic Amino Acids) Glycine	*l*-Alanine	*l*-Valine	*l*-Leucine	*l*-Isoleucine
0	0	0	920,000	800,000	260,000	920,000	260,000
0	0	5	560,000	520,000	380,000	740,000	340,000
0	0	0.5	700,000	660,000	460,000	960,000	400,000
0	0	0.05	480,000	520,000	180,000	460,000	460,000
0	0.6 Units	0	760,000	1,140,000	420,000	740,000	420,000
0	0.6 Units	5	440,000	980,000	440,000	1,000,000	340,000
0	0.6 Units	0.5	300,000	780,000	300,000	920,000	400,000
0	0.6 Units	0.05	580,000	760,000	340,000	700,000	520,000
20 pmol MPO	0.6 Units	0	500,000	700,000	300,000	1,640,000	300,000
20 pmol MPO	0.6 Units	5	0	0	34,000	72,000	22,000
20 pmol MPO	0.6 Units	0.5	0	0	2,000	42,000	0
20 pmol MPO	0.6 Units	0.05	260,000	4,000	16,000	62,000	10,000
20 pmol EPO	0.6 Units	0	780,000	840,000	200,000	1,060,000	200,000
20 pmol EPO	0.6 Units	5	0	0	0	52,000	0
20 pmol EPO	0.6 Units	0.5	0	0	0	0	0
20 pmol EPO	0.6 Units	0.05	182,000	10,000	28,000	0	30,000

As shown in **Table 2**, each of the aliphatic amino acids tested exhibited a significant enhancing effect on the haloperoxidase antimicrobial activity of both eosinophil peroxidase and myeloperoxidase against *A. fumigatus* spores, with glycine and *l*-alanine exhibiting the greatest enhancing effect.

The dicarboxylic amino acids and amides *l*-aspartic acid, *l*-asparagine, *l*-glutamic acid and *l*-glutamine, and the imino acids *l*-proline and *l*-hydroxyproline were tested as described above. The results are shown in the following **Table 3**:

Table 3
Amino Acid Type and Concentration: Effect on Haloperoxidase Killing of *Aspergillus fumigatus* Spores

Haloperoxidase	Glucose Oxidase	(Amino Acid) µmol/ml (mM)	CFU (Dicarboxylic Amino Acids & Amides)				CFU (Imino Acids)	
			l-Aspartic Acid	*l*-Asparagine	*l*-Glutamic Acid	*l*-Glutamine	*l*-Proline	*l*-Hydroxyproline
0	0	0	520,000	520,000	920,000	920,000	260,000	860,000
0	0	5	540,000	540,000	260,000	420,000	420,000	640,000
0	0	0.5	460,000	180,000	320,000	660,000	300,000	800,000
0	0	0.05	200,000	240,000	580,000	300,000	480,000	740,000
0	0.6 Units	0	340,000	340,000	740,000	740,000	420,000	740,000
0	0.6 Units	5	420,000	340,000	280,000	400,000	240,000	540,000
0	0.6 Units	0.5	360,000	460,000	340,000	360,000	360,000	460,000
0	0.6 Units	0.05	380,000	160,000	640,000	560,000	200,000	720,000
20 pmol MPO	0.6 Units	0	700,000	700,000	1,640,000	1,640,000	300,000	940,000
20 pmol MPO	0.6 Units	5	640,000	660,000	840,000	1,080,000	540,000	1,000,000
20 pmol MPO	0.6 Units	0.5	960,000	340,000	820,000	1,000,000	380,000	900,000
20 pmol MPO	0.6 Units	0.05	800,000	680,000	820,000	750,000	280,000	680,000
20 pmol EPO	0.6 Units	0	920,000	920,000	1,060,000	1,060,000	200,000	860,000
20 pmol EPO	0.6 Units	5	920,000	1,340,000	1,100,000	820,000	280,000	840,000
20 pmol EPO	0.6 Units	0.5	1,460,000	440,000	540,000	660,000	460,000	1,260,000
20 pmol EPO	0.6 Units	0.05	620,000	1,260,000	900,000	400,000	380,000	680,000

As shown in **Table 3**, none of the dicarboxylic amino acids or imino acids tested exhibited a significant haloperoxidase antimicrobial activity enhancing effect at any of the concentrations tested.

The hydroxyamino acids *l*-serine and *l*-threonine, and the basic amino acids *l*-lysine, *l*-histidine and *l*-arginine were tested as described above. The results are shown in the following **Table 4**:

Table 4

Amino Acid Type and Concentration: Effect on Haloperoxidase Killing of *Aspergillus fumigatus* Spores

Haloperoxidase	Glucose Oxidase	(Amino Acid) µmol/ml (mM)	CFU (Hydroxyamino Acids) *l*-Serine	CFU (Hydroxyamino Acids) *l*-Threonine	CFU (Basic Amino Acids) *l*-Lysine	CFU (Basic Amino Acids) *l*-Histidine	CFU (Basic Amino Acids) *l*-Arginine
0	0	0	800,000	760,000	520,000	760,000	800,000
0	0	5	820,000	520,000	520,000	440,000	780,000
0	0	0.5	540,000	800,000	460,000	840,000	740,000
0	0	0.05	580,000	660,000	460,000	840,000	620,000
0	0.6 Units	0	1,140,000	400,000	340,000	400,000	1,140,000
0	0.6 Units	5	480,000	800,000	240,000	520,000	580,000
0	0.6 Units	0.5	620,000	360,000	480,000	740,000	960,000
0	0.6 Units	0.05	640,000	560,000	520,000	560,000	1,020,000
20 pmol MPO	0.6 Units	0	700,000	500,000	700,000	500,000	700,000
20 pmol MPO	0.6 Units	5	700,000	400,000	380,000	720,000	960,000
20 pmol MPO	0.6 Units	0.5	660,000	0	0	640,000	740,000
20 pmol MPO	0.6 Units	0.05	560,000	12,000	0	520,000	840,000
20 pmol EPO	0.6 Units	0	840,000	780,000	920,000	780,000	840,000
20 pmol EPO	0.6 Units	5	0	1,000,000	560,000	740,000	960,000
20 pmol EPO	0.6 Units	0.5	34,000	0	620,000	600,000	700,000
20 pmol EPO	0.6 Units	0.05	860,000	40,000	920,000	102,000	900,000

As shown in **Table 4**, the hydroxyamino acids *l*-serine and *l*-threonine both significantly enhanced eosinoperoxidase killing of *A. fumigatus* spores while *l*-threonine and the basic amino acid *l*-lysine were effective in enhancing myeloperoxidase antimicrobial activity. The basic amino acids *l*-histidine and *l*-arginine exhibited no significant antimicrobial effects. Histidine is very reactive with singlet molecular oxygen, and as such, it would be expected to produce potent competitive inhibition of haloperoxidase action which might mask its capacity to stimulate spore germination.

The sulfur amino acids *l*-cysteine and *l*-methionine, and the aromatic amino acids *l*-phenylalanine, *l*-tyrosine and *l*-tryptophan were tested as described above. The results are shown in the following **Table 5**:

Table 5
Amino Acid Type and Concentration: Effect on Haloperoxidase Killing of *Aspergillus fumigatus* Spores

Haloperoxidase	Glucose Oxidase	(Amino Acid) µmol/ml (mM)	CFU (Sulfur Amino Acids) *l*-Cysteine	CFU (Sulfur Amino Acids) *l*-Methionine	CFU (Aromatic Amino Acids) *l*-Phenylalanine	CFU (Aromatic Amino Acids) *l*-Tyrosine	CFU (Aromatic Amino Acids) *l*-Tryptophan
0	0	0	380,000	380,000	260,000	760,000	380,000
0	0	5	540,000	560,000	360,000	580,000	800,000
0	0	0.5	500,000	540,000	340,000	800,000	640,000
0	0	0.05	380,000	320,000	380,000	560,000	700,000
0	0.6 Units	0	460,000	460,000	420,000	400,000	460,000
0	0.6 Units	5	580,000	660,000	400,000	840,000	920,000
0	0.6 Units	0.5	420,000	480,000	460,000	700,000	580,000
0	0.6 Units	0.05	360,000	460,000	440,000	560,000	280,000
20 pmol MPO	0.6 Units	0	580,000	580,000	300,000	500,000	580,000
20 pmol MPO	0.6 Units	5	580,000	400,000	8,000	640,000	700,000
20 pmol MPO	0.6 Units	0.5	540,000	480,000	4,000	460,000	580,000
20 pmol MPO	0.6 Units	0.05	720,000	560,000	2,000	20,000	1,040,000
20 pmol EPO	0.6 Units	0	480,000	480,000	200,000	780,000	480,000
20 pmol EPO	0.6 Units	5	680,000	580,000	0	640,000	860,000
20 pmol EPO	0.6 Units	0.5	800,000	560,000	0	920,000	740,000
20 pmol EPO	0.6 Units	0.05	240,000	600,000	0	0	580,000

As shown in **Table 5**, the sulfur amino acids were ineffective in enhancing antimicrobial activity of either eosinophil peroxidase or myeloperoxidase. The aromatic amino acids *l*-phenylalanine and *l*-tyrosine both exhibited significant enhancement of eosinophil peroxidase and myeloperoxidase killing of *A. fumigatus*, while *l*-tryptophan exhibited no significant effect. These sulfur and aromatic amino acids are also relatively reactive with singlet molecular oxygen, and may competitively inhibit haloperoxidase action which would mask their capacity to stimulate spore germination. This might explain why *l*-phenylalanine and *l*-tyrosine are most effective when tested at a low concentration.

The effect of enantiomeric configuration of alanine and of isomeric configuration and derivatisation of alanine were tested as described above. The results are shown in the following **Table 6**:

Table 6
Amino Acid Type and Concentration: Effect on Haloperoxidase Killing of *Aspergillus fumigatus* Spores

Haloperoxidase	Glucose Oxidase	(Amino Acid) μmol/ml (mM)	CFU (Alinine Isomers and Derivatives) *l*-Alanine	*d*-Alanine	β-Alanine	*l*-Ala Methyl Ester	*l*-Ala-*l*-Ala
0	0	0	800,000	740,000	740,000	740,000	740,000
0	0	5	520,000	720,000	580,000	500,000	660,000
0	0	0.5	660,000	760,000	920,000	540,000	640,000
0	0	0.05	520,000	760,000	740,000	540,000	620,000
0	0.6 Units	0	1,140,000	760,000	760,000	760,000	760,000
0	0.6 Units	5	980,000	660,000	900,000	860,000	780,000
0	0.6 Units	0.5	780,000	700,000	620,000	740,000	680,000
0	0.6 Units	0.05	760,000	600,000	860,000	1,200,000	360,000
20 pmol MPO	0.6 Units	0	700,000	820,000	820,000	820,000	820,000
20 pmol MPO	0.6 Units	5	0	14,000	760,000	0	660,000
20 pmol MPO	0.6 Units	0.5	0	0	900,000	0	500,000
20 pmol MPO	0.6 Units	0.05	4,000	10,000	1,116,000	440,000	480,000
20 pmol EPO	0.6 Units	0	840,000	1,020,000	1,020,000	1,020,000	1,020,000
20 pmol EPO	0.6 Units	5	0	0	940,000	0	660,000
20 pmol EPO	0.6 Units	0.5	0	0	880,000	0	1,360,000
20 pmol EPO	0.6 Units	0.05	10,000	10,000	720,000	580,000	680,000

As shown in **Table 6**, both the *l*- and *d*-enantiomers of alanine were highly effective in enhancing the myeloperoxidase and eosinophil peroxidase killing of *A. fumigatus*, while β-alanine exhibited no significant enhancing effect. Similarly, the methyl ester of *l*-alanine produced significant enhancement of antimicrobial activity. The *l*-alanine-*l*-alanine dipeptide exhibited no significant enhancement activity.

The effect of enantiomeric configuration of threonine was also tested as described above. The results are shown in the following **Table 7**:

Table 7

Amino Acid Type and Concentration: Effect on Haloperoxidase Killing of *Aspergillus fumigatus* Spores

Haloperoxidase	Glucose Oxidase	(Amino Acid) μmol/ml (mM)	CFU (Enantiomers of Hydroxyamino Acids) *l*-Threonine	*d*-Threonine
0	0	0	760,000	800,000
0	0	5	520,000	820,000
0	0	0.5	800,000	760,000
0	0	0.05	660,000	680,000
0	0.6 Units	0	400,000	1,140,000
0	0.6 Units	5	800,000	460,000
0	0.6 Units	0.5	360,000	720,000
0	0.6 Units	0.05	560,000	720,000
20 pmol MPO	0.6 Units	0	500,000	700,000
20 pmol MPO	0.6 Units	5	400,000	420,000
20 pmol MPO	0.6 Units	0.5	0	0
20 pmol MPO	0.6 Units	0.05	12,000	280,000
20 pmol EPO	0.6 Units	0	780,000	840,000
20 pmol EPO	0.6 Units	5	1,000,000	0
20 pmol EPO	0.6 Units	0.5	0	0
20 pmol EPO	0.6 Units	0.05	40,000	520,000

As shown in **Table 7**, both the *l*- and *d*-enantiomers of threonine significantly enhanced the myeloperoxidase and eosinophil peroxidase killing of *A. fumigatus*.

The effect of using the α-keto acid forms of enhancing α-amino acids was tested with *l*-alanine and pyruvic acid, and with glycine and glyoxylic acid, as described above. The results are shown in the following **Table 8**:

Table 8
Amino Acid Type and Concentration: Effect on Haloperoxidase Killing of *Aspergillus fumigatus* Spores

Haloperoxidase	Glucose Oxidase	(Amino Acid) µmol/ml (mM)	CFU (Amino Acid versus Alpha Keto Acid)			
			l-Alanine	Pyruvic Acid	Glycine	Glyoxylic Acid
0	0	0	800,000	860,000	860,000	860,000
0	0	5	520,000	880,000	720,000	600,000
0	0	0.5	660,000	700,000	480,000	700,000
0	0	0.05	520,000	660,000	640,000	560,000
0	0.6 Units	0	1,140,000	740,000	740,000	740,000
0	0.6 Units	5	980,000	760,000	520,000	560,000
0	0.6 Units	0.5	780,000	600,000	760,000	600,000
0	0.6 Units	0.05	760,000	480,000	800,000	660,000
20 pmol MPO	0.6 Units	0	700,000	940,000	940,000	940,000
20 pmol MPO	0.6 Units	5	0	820,000	0	1,060,000
20 pmol MPO	0.6 Units	0.5	0	880,000	0	860,000
20 pmol MPO	0.6 Units	0.05	4,000	580,000	90,000	580,000
20 pmol EPO	0.6 Units	0	840,000	860,000	860,000	860,000
20 pmol EPO	0.6 Units	5	0	640,000	0	740,000
20 pmol EPO	0.6 Units	0.5	0	560,000	0	720,000
20 pmol EPO	0.6 Units	0.05	10,000	780,000	460,000	740,000

As shown in **Table 8**, pyruvic acid and glyoxylic acid do not exhibit the activity enhancing effect of *l*-alanine and glycine.

Example 3

Effect of Antimicrobial Activity Enhancing Agents on Other Antifungal Systems

In order to determine the effect of *l*-alanine on the antimicrobial effect of the common antifungal compounds nystatin and amphotericin B, the procedure of Example 2 was followed using nystatin or amphotericin B in place of the haloperoxidase-glucose oxidase of Example 2 and 0 (control) or 10 μmol/ml of *l*-alanine with *Fusarium moniliforme* as the microbe. The results are shown in the following **Table 9**:

Table 9

Effect of *l*-Alanine on Nystatin and Amphotericin B Antifungal Activity

Antifungal Agent Final Concentration	*l*-Alanine μmol/test	CFU (*F. moniliforme* ATCC 38159)
None	None	940,000
Nystatin, 400 μg/ml	None	120,000
Nystatin, 40 μg/ml	None	340,000
Nystatin, 4 μg/ml	None	500,000
None	10	1,020,000
Nystatin, 400 μg/ml	10	28,000
Nystatin, 40 μg/ml	10	124,000
Nystatin, 4 μg/ml	10	220,000
None	None	880,000
Amphotericin B, 250 μg/ml	None	320,000
Amphotericin B, 25 μg/ml	None	500,000
Amphotericin B, 2.5 μg/ml	None	880,000
None	10	840,000
Amphotericin B, 250 μg/ml	10	340,000
Amphotericin B, 25 μg/ml	10	460,000
Amphotericin B, 2.5 μg/ml	10	800,000

As can be seen in **Table 9**, the addition of *l*-alanine doubled the nystatin-dependent killing of *Fusarium moniliforme* but had no effect on amphotericin B-dependent killing of *Fusarium moniliforme*.

The foregoing procedure was repeated using the antiseptic compounds hydrogen peroxide (H_2O_2) and sodium hypochlorite (NaClO) with the spores of

Aspergillus fumigatus and *Fusarium moniliforme*. The results are shown in the following **Table 10**:

Table 10

Effect of *l*-Alanine on Hydrogen Peroxide and Sodium Hypochlorite Antifungal Activity

Antifungal Agent Final Concentration	*l*-Alanine μmol/test	CFU (*A. fumigatus* ATCC 10894)	CFU (*F. moniliforme* ATCC 38159)
None	None	360,000	460,000
H_2O_2, 3 mg/ml	None	580,000	0
H_2O_2, 0.3 mg/ml	None	400,000	140,000
H_2O_2, 0.03 mg/ml	None	440,000	400,000
H_2O_2, 0.003 mg/ml	None	640,000	500,000
None	10	700,000	580,000
H_2O_2, 3 mg/ml	10	720,000	0
H_2O_2, 0.3 mg/ml	10	280,000	340,000
H_2O_2, 0.03 mg/ml	10	660,000	300,000
H_2O_2, 0.003 mg/ml	10	500,000	310,000
None	None	300,000	284,000
NaClO, 1 mg/ml	None	10,000	50,000
NaClO, 0.1 mg/ml	None	26,000	298,000
NaClO 0.01 mg/ml	None	560,000	290,000
NaClO, 0.001 mg/ml	None	640,000	294,000
None	10	700,000	380,000
NaClO, 1 mg/ml	10	26,000	292,000
NaClO, 0.1 mg/ml	10	54,000	404,000
NaClO, 0.01 mg/ml	10	580,000	404,000
NaClO, 0.001 mg/ml	10	280,000	304,000

As with amphotericin B, no significant enhancement of hydrogen peroxide or hypochlorite antisepsis is seen when used in combination with *l*-alanine. In fact, *l*-alanine appears to inhibit peroxide and especially hypochlorite killing of the fungi. Under such conditions *l*-alanine probably acts as a competitive inhibitor.

Example 4

Effect of *l*-Alanine on Haloperoxidase Killing of Additional Organisms

The effect of *l*-alanine on the halperoxidase antimicrobial activity against *Fusarium moniliforme*, *Tricophyton rubrum* and *Crytococcus neoformans* was determined following the procedure of Example 2 using 0 (control) or 10 μmol/ml of

l-alanine and 2, 10 or 50 pmol of myeloperoxidase or eosinophil peroxidase per test against these organisms. The results are shown in the following **Table 11** (*F. moniliforme*, ATCC # 38159), **Table 12** (*T. rubrum*, ATCC #28188, 18753 and 18758) and **Table 13** (*C. neoformans*, ATCC #14115):

Table 11

Haloperoxidase Killing of *Fusarium moniliforme*

Haloperoxidase	Glucose Oxidase	*l*-Alanine µmol/test	*Fusarium moniliforme* ATCC #38159
None	None	10	1,720,000
None	0.6 Units	None	1,380,000
None	0.6 Units	10	1,760,000
50 pmol MPO	None	10	1,640,000
50 pmol MPO	0.6 Units	None	0
10 pmol MPO	0.6 Units	None	0
50 pmol MPO	0.6 Units	10	0
10 pmol MPO	0.6 Units	10	8,000
50 pmol EPO	None	10	8,000
50 pmol EPO	0.6 Units	None	0
10 pmol EPO	0.6 Units	None	0
2 pmol EPO	0.6 Units	None	0
50 pmol EPO	0.6 Units	10	0
10 pmol EPO	0.6 Units	10	0
2 pmol EPO	0.6 Units	10	0

Table 12
Haloperoxidase Killing of *Trichophyton*

Haloperoxidase	Glucose Oxidase	*l*-Alanine µmol/test	*Trichophyton rubrum* ATCC #28188	*Trichophyton rubrum* ATCC #18753	*Trichophyton rubrum* ATCC #18758
None	None	10	1,700,000	200,000	1,440,000
None	0.6 Units	10	1,580,000	276,000	128,000
50 pmol MPO	None	10	2,680,000	192,000	720,000
50 pmol MPO	0.6 Units	None	1,240,000	132,000	414,000
50 pmol MPO	0.6 Units	10	84,000	18,000	0
10 pmol MPO	0.6 Units	10	184,000	62,000	0
2 pmol MPO	0.6 Units	10	1,380,000	310,000	4,000
50 pmol EPO	None	10	3,600,000	332,000	1,800,000
50 pmol EPO	0.6 Units	None	1,180,000	22,000	244,000
50 pmol EPO	0.6 Units	10	0	0	0
10 pmol EPO	0.6 Units	10	0	0	0
2 pmol EPO	0.6 Units	10	0	0	0

Table 13
Haloperoxidase Killing of *Cryptococcus neoformans*

Haloperoxidase	Glucose Oxidase	*l*-Alanine µmol/test	CFU (*Cryptococcus neoformans*) ATCC #14115)
None	None	10	960,000
None	0.6 Units	None	680,000
None	0.6 Units	10	480,000
50 pmol MPO	None	10	480,000
50 pmol MPO	0.6 Units	None	480,000
50 pmol MPO	0.6 Units	10	440,000
10 pmol MPO	0.6 Units	10	860,000
2 pmol MPO	0.6 Units	10	220,000
50 pmol EPO	None	10	660,000
50 pmol EPO	0.6 Units	None	460,000
50 pmol EPO	0.6 Units	10	0
10 pmol EPO	0.6 Units	10	0
2 pmol EPO	0.6 Units	10	640,000

As shown in **Table 11**, myeloperoxidase and eosinophil peroxidase are both highly effective against *Fusarium moniliforme* either in the presence or absence of *l*-alanine. In fact, EPO was found to be effective in the absence of glucose oxidase. As shown in **Table 12**, complete killing of *Trichophyton* is obtained with eosinophil peroxidase in the presence of *l*-alanine, while a significant enhancement of myeloperoxidase killing is obtained.

As shown in **Table 13**, *l*-alanine also significantly enhances eosinophil peroxidase killing of *Cryptococcus neoformans*, while some enhancement in myeloperoxidase activity is seen.

Example 5

Effect of Cholesterol Oxidase Concentration on Oxidase-Haloperoxidase Microbicidal Action Against *Candida albicans*

The effect of cholesterol oxidase concentration on oxidase-haloperoxidase microbicidal action against Candida albicans was determined by following the procedure of Example 1, except that each test contained a different quantity of cholesterol oxidase from *Nocardia erythropolis* (0.1 unit = 4 µg) as indicated,

10 pmol (1.4 µg) porcine MPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1899201) or 10 pmol (0.7 µg) porcine EPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1929201) in 50 mM Acetate Buffer containing 100 mEq/L Cl^-, 1 mEq/L Br^-, and 1 mM *l*-alanine. The pH was 6.7 with 50 mM MOPS as buffer. The final suspension contained 7 mM (7 µmol/ml) cholesterol in 8.5% ethanol. The final volume was 1 ml. After two hours incubation at 37°C, the microbes were plated on Sabouraud's dextrose agar. The results are expressed in **Table 14** as the colony forming units (CFU) counted.

Table 14

Effect of Cholesterol Oxidase Concentration on Oxidase-Haloperoxidase Microbicidal Action Against *Candida albicans*:

Organism	Cholesterol Oxidase	Haloperoxidase	CFU
Cand. albicans	None	None	400,000
Cand. albicans	0.1 Unit	None	480,000
Cand. albicans	0.1 Unit †	10 pmol MPO	420,000
Cand. albicans	0.1 Unit	10 pmol MPO	0
Cand. albicans	0.05 Unit	10 pmol MPO	0
Cand. albicans	0.025 Unit	10 pmol MPO	0
Cand. albicans	0.013 Unit	10 pmol MPO	76,000
Cand. albicans	None	10 pmol MPO	560,000
Cand. albicans	0.1 Unit †	10 pmol EPO	400,000
Cand. albicans	0.1 Unit	10 pmol EPO	0
Cand. albicans	0.05 Unit	10 pmol EPO	0
Cand. albicans	0.025 Unit	10 pmol EPO	200
Cand. albicans	0.013 Unit	10 pmol EPO	180,000
Cand. albicans	None	10 pmol EPO	360,000

† indicates that the 50 mM Acetate Buffer did NOT contain *l*-alanine.

As shown in **Table 14**, complete killing of *C. albicans* was observed with 0.025 unit cholesterol oxidase plus 10 pmol MPO and 1 mM *l*-alanine. In the absence of *l*-alanine, no killing was observed with 0.1 unit cholesterol oxidase with 10 pmol MPO. Similar results were obtained when EPO was substituted as the haloperoxidase.

Example 6

Effect of Cholesterol Oxidase Concentration on Oxidase-Haloperoxidase Microbicidal Action Against Bacteria

The procedure of Example 5 was followed using *Escherichia coli* in place of the *C. albicans* of Example 5. Each test contained the indicated quantity of cholesterol oxidase from *Nocardia erythropolis* (0.1 unit = 4 μg), 10 pmol (1.4 μg) porcine MPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1899201) or 10 pmol (0.7 μg) porcine EPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1929201) in 50 mM Acetate Buffer containing 100 mEq/L Cl^-, 1 mEq/L Br^-, and 1 mM *l*-alanine. The pH was 6.7 with 50 mM MOPS as buffer. The final suspension contained 7 mM (7 μmol/ml) cholesterol in 8.5% ethanol. The final volume was 1 ml. After two hours incubation at 37°C, the microbes were plated on trypticase soy agar. The results are expressed in the following **Table 15** as the colony forming units (CFU) counted.

Table 15

Effect of Cholesterol Oxidase Concentration on Oxidase-Haloperoxidase Microbicidal Action Against *Escherichia coli*:

Organism	Cholesterol Oxidase	Haloperoxidase	CFU
Escherichia coli	None	None	13,500,000
Escherichia coli	0.1 Unit	None	2,300,000
Escherichia coli	0.1 Unit †	10 pmol MPO	0
Escherichia coli	0.1 Unit	10 pmol MPO	0
Escherichia coli	0.05 Unit	10 pmol MPO	0
Escherichia coli	0.025 Unit	10 pmol MPO	0
Escherichia coli	0.013 Unit	10 pmol MPO	
Escherichia coli	None	10 pmol MPO	14,000,000
Escherichia coli	0.1 Unit †	10 pmol EPO	0
Escherichia coli	0.1 Unit	10 pmol EPO	0
Escherichia coli	0.05 Unit	10 pmol EPO	0
Escherichia coli	0.025 Unit	10 pmol EPO	0
Escherichia coli	0.013 Unit	10 pmol EPO	0
Escherichia coli	None	10 pmol EPO	8,200,000

† indicates that the 50 mM Acetate Buffer did NOT contain *l*-alanine.

As shown in **Table 15**, complete MPO and EPO killing of *Escherichia coli* was observed at all cholesterol oxidase concentrations tested (0.0125 to 0.1 unit) in

the absence or presence of 1 mM *l*-alanine. The same results were observed with *Staphylococcus aureus* (data not shown).

Example 7

Oxidase-Haloperoxidase Killing of Bacterial, Yeast and Fungal Spores Using Choline Oxidase

The procedure of Example 1 was followed except that 0.2 unit choline oxidase was employed as the H_2O_2-generating oxidase. Where indicated the reaction contained 0.2 unit (i.e., 20 μg) choline oxidase from *Alcaligenes sp.*, 20 pmol (2.8 μg) porcine MPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1899201) or 20 pmol (1.5 μg) porcine EPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1929201) in 50 mM Acetate Buffer containing 100 mEq/L Cl^-, and 1 mEq/L Br^-. The pH was 7 with 50 mM MOPS as buffer. The final concentration of choline was 150 mM (150 μmol/ml). The final volume was 1 ml. After four hours incubation at 22°C the microbes were plated (*S. aureus* was plated on trypticase soy agar; *C. albicans* and *A. fumigatus* were plated on Sabouraud's dextrose agar). The results are expressed in **Table 16** as the colony forming units (CFU) counted.

Table 16

Effect of *l*-Alanine on Choline Oxidase-Haloperoxidase Killing of *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus fumigatus* Spores:

Organism	Choline Oxidase	Haloperoxidase	CFU
Staph. aureus	None	None	19,400,000
Staph. aureus	0.2 Unit	None	13,800,000
Staph. aureus	0.2 Unit †	None	15,400,000
Staph. aureus	0.2 Unit	20 pmol MPO	0
Staph. aureus	0.2 Unit †	20 pmol MPO	0
Staph. aureus	0.2 Unit	20 pmol EPO	0
Staph. aureus	0.2 Unit †	20 pmol EPO	0
Cand. albicans	None	None	1,460,000
Cand. albicans	0.2 Unit	None	1,200,000
Cand. albicans	0.2 Unit †	None	1,180,000
Cand. albicans	0.2 Unit	20 pmol MPO	1,120,000
Cand. albicans	0.2 Unit †	20 pmol MPO	0
Cand. albicans	0.2 Unit	20 pmol EPO	640,000
Cand. albicans	0.2 Unit †	20 pmol EPO	0
Asperg. fumigatus	None	None	1,260,000
Asperg. fumigatus	0.2 Unit	None	1,260,000
Asperg. fumigatus	0.2 Unit †	None	1,300,000
Asperg. fumigatus	0.2 Unit	20 pmol MPO	800,000
Asperg. fumigatus	0.2 Unit †	20 pmol MPO	0
Asperg. fumigatus	0.2 Unit	20 pmol EPO	740,000
Asperg. fumigatus	0.2 Unit †	20 pmol EPO	0

† indicates that the 50 mM Acetate Buffer contained 1 mM *l*-alanine.

Example 8

Oxidase-Haloperoxidase Killing of Bacterial, Yeast and Fungal Spores Using Lactate Oxidase

The procedure of Example 7 was followed except that 0.2 unit lactate oxidase was employed as the H_2O_2-generating oxidase. Where indicated the reaction contained 0.2 unit (i.e., 5 μg) lactate oxidase from *Pediococcus sp.*, 20 pmol (2.8 μg) porcine MPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1899201) or 20 pmol (1.5 μg) porcine EPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1929201) in 50 mM Acetate Buffer containing 100 mEq/L Cl^-, and 1 mEq/L Br^-. The pH was 7 with 50 mM MOPS as buffer. The final concentration of lactate was 150 mM (150 μmol/ml). The final volume was 1 ml. After four hours incubation

(22°C) the microbes were plated. *S. aureus* was plated on trypticase soy agar. *C. albicans* and *A. fumigatus* were plated on Sabouraud's dextrose agar. The results are expressed in **Table 17** as the colony forming units (CFU) counted.

Table 17

Effect of *l*-Alanine on Lactate Oxidase-Haloperoxidase Killing of *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus fumigatus* Spores:

Organism	Lactate Oxidase	Haloperoxidase	CFU
Staph. aureus	None	None	19,400,000
Staph. aureus	0.2 Unit	None	23,400,000
Staph. aureus	0.2 Unit †	None	24,400,000
Staph. aureus	0.2 Unit	20 pmol MPO	0
Staph. aureus	0.2 Unit †	20 pmol MPO	0
Staph. aureus	0.2 Unit	20 pmol EPO	0
Staph. aureus	0.2 Unit †	20 pmol EPO	0
Cand. albicans	None	None	1,460,000
Cand. albicans	0.2 Unit	None	1,480,000
Cand. albicans	0.2 Unit †	None	1,020,000
Cand. albicans	0.2 Unit	20 pmol MPO	1,380,000
Cand. albicans	0.2 Unit †	20 pmol MPO	1,500,000
Cand. albicans	0.2 Unit	20 pmol EPO	1,400,000
Cand. albicans	0.2 Unit †	20 pmol EPO	1,180,000
Asperg. fumigatus	None	None	1,260,000
Asperg. fumigatus	0.2 Unit	None	860,000
Asperg. fumigatus	0.2 Unit †	None	760,000
Asperg. fumigatus	0.2 Unit	20 pmol MPO	740,000
Asperg. fumigatus	0.2 Unit †	20 pmol MPO	400,000
Asperg. fumigatus	0.2 Unit	20 pmol EPO	760,000
Asperg. fumigatus	0.2 Unit †	20 pmol EPO	18,000

† indicates that the 50 mM Acetate Buffer contained 1 mM *l*-alanine.

Example 9

Oxidase-Haloperoxidase Killing of Bacterial, Yeast and Fungal Spores Using Alcohol Oxidase

The procedure of Example 7 was followed except that 0.2 unit alcohol oxidase was employed as the H_2O_2-generating oxidase. Where indicated the reaction contained 0.2 unit (i.e., 21 μg) alcohol oxidase from *Candida boidinii*, 20 pmol (2.8 μg) porcine MPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1899201) or 20 pmol (1.5 μg) porcine EPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A.,

Lot#1929201) in 50 mM Acetate Buffer containing 100 mEq/L Cl^-, and 1 mEq/L Br^-. The pH was 7 with 50 mM MOPS as buffer. The final concentration of alcohol was 150 mM (150 μmol/ml). The final volume was 1 ml. After four hours incubation at 22°C the microbes were plated. *S. aureus* was plated on trypticase soy agar. *C. albicans* and *A. fumigatus* were plated on Sabouraud's dextrose agar. The results are expressed in **Table 18** as the colony forming units (CFU) counted.

Table 18

Effect of *l*-Alanine on Alcohol Oxidase-Haloperoxidase Killing of *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus fumigatus* Spores:

Organism	Alcohol Oxidase	Haloperoxidase	CFU
Staph. Aureus	None	None	19,400,000
Staph. Aureus	0.2 Unit	None	21,200,000
Staph. Aureus	0.2 Unit †	None	19,400,000
Staph. Aureus	0.2 Unit	20 pmol MPO	840,000
Staph. Aureus	0.2 Unit †	20 pmol MPO	760,000
Staph. Aureus	0.2 Unit	20 pmol EPO	0
Staph. Aureus	0.2 Unit †	20 pmol EPO	0
Cand. Albicans	None	None	1,460,000
Cand. Albicans	0.2 Unit	None	1,100,000
Cand. Albicans	0.2 Unit †	None	1,460,000
Cand. Albicans	0.2 Unit	20 pmol MPO	1,180,000
Cand. Albicans	0.2 Unit †	20 pmol MPO	1,280,000
Cand. Albicans	0.2 Unit	20 pmol EPO	1,220,000
Cand. Albicans	0.2 Unit †	20 pmol EPO	1,160,000
Asperg. fumigatus	None	None	1,260,000
Asperg. fumigatus	0.2 Unit	None	620,000
Asperg. fumigatus	0.2 Unit †	None	700,000
Asperg. fumigatus	0.2 Unit	20 pmol MPO	1,560,000
Asperg. fumigatus	0.2 Unit †	20 pmol MPO	320,000
Asperg. fumigatus	0.2 Unit	20 pmol EPO	1,040,000
Asperg. fumigatus	0.2 Unit †	20 pmol EPO	16,000

† indicates that the 50 mM Acetate Buffer contained 1 mM *l*-alanine.

Cholesterol oxidase (**Table 1**), choline oxidase (**Table 16**), and lactate oxidase (**Table 17**) in combination with either MPO or EPO produced complete killing of *Staphylococcus aureus* in the presence or absence of *l*-alanine. With alcohol oxidase (**Table 18**), killing was complete with EPO, but only partial with MPO either with or without *l*-alanine.

Candida albicans was totally killed by cholesterol oxidase (**Table 1**) and choline oxidase (**Table 16**) plus MPO or EPO only in the presence of *l*-alanine. In the absence of *l*-alanine, microbicidal action was limited to approximately fifty percent kill. Lactate oxidase (**Table 17**) and alcohol oxidase (**Table 18**) were not effective in driving MPO or EPO killing of *Candida albicans* in the presence or absence of *l*-alanine.

In the presence of *l*-alanine, both cholesterol oxidase (**Table 1**) and choline oxidase (**Table 16**) supported complete killing of *Aspergillus fumigatus* spores by MPO or EPO. However, only partial killing was observed in the absence of *l*-alanine. Although incomplete, both lactate oxidase (**Table 17**) and alcohol oxidase (**Table 18**) produced greater than 90% spore kill with EPO and greater than 50% spore kill with MPO in the presence of *l*-alanine. In the absence of *l*-alanine, neither lactate oxidase or alcohol oxidase supported MPO or EPO killing of *Aspergillus fumigatus* spores.

Example 10

The Effect of Potential Activator Substances on Cholesterol Oxidase-Haloperoxidase Killing of Bacillus and Aspergillus Spores.

The microbiology literature describes several substances that might serve as activators of germination of spores and vegetative forms. Conidiospore germination has been reported to require glucose, phosphate and an amino acid. *L*-proline or *l*-alanine fulfill the amino acid requirement. Other amino acids and vitamins are less effective (Yanigita, 1957, *Arch Mikrobiol* **26**:329).

Several other organic compounds have been reported to stimulate germination. These include phenethyl alcohol (Lingappa et al., 1970, *Arch Mikrobiol* **72**:97), coumarin (Weber and Hess, 1974, *The Fungal Spore*, p.178, Wiley-Interscience), and furfural derivatives (Sussman et al., 1959, *Mycologia* **51**:237).

The effect of *l*-alanine and *l*-proline on cholesterol oxidase-haloperoxidase killing of *Bacillus cereus* endospores contained 0.1 unit (i.e., 4 µg) cholesterol oxidase from *Nocardia erythropolis*, 20 pmol (2.8 µg) porcine MPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1899201) or 20 pmol (1.5 µg) porcine EPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1929201), and the indicated quantity of *l*-alanine or *l*-proline in 50 mM Acetate Buffer containing 100 mEq/L Cl^-, and 1 mEq/L Br^-. The pH was adjusted to ~7 by addition of 50 mM MOPS buffer. The final concentration of cholesterol was 5 mM (5 µmol/ml) in 8.5% ethanol. The final volume was 1 ml. After the indicated period of incubation (22°C), the surviving microbes were plated on trypticase soy agar. The results are shown in **Table 19** as the colony forming units (CFU) counted.

Table 19

Effects of *l*-Alanine and *l*-Proline on Cholesterol Oxidase-Haloperoxidase Microbicidal Action Against *Bacillus cereus* Spores:

Treatment:		*Bacillus cereus* Survival, CFU (Post Exposure Time in Hours)				
Haloperoxidase	Activator Substance	0.5 Hr.	1 Hr.	1.5 Hr.	2 Hrs.	3 Hrs.
None	None	900,000	460,000	720,000	720,000	520,000
None	*l*-alanine, 10 μmol	860,000	660,000	560,000	520,000	400,000
None	*l*-proline, 10 μmol	820,000	760,000	620,000	320,000	540,000
MPO, 20 pmol	None	0	0	0	0	0
MPO, 20 pmol	*l*-alanine, 10 μmol	0	0	0	0	0
MPO, 20 pmol	*l*-proline, 10 μmol	0	0	0	0	0
EPO, 20 pmol	None	2,000	2,000	0	0	0
EPO, 20 pmol	*l*-alanine, 10 μmol	2,000	0	0	0	0
EPO, 20 pmol	*l*-proline, 10 μmol	0	2,000	0	0	0

After thirty minutes of incubation, cholesterol oxidase (0.1 unit) with MPO (20 pmol) produced complete killing of *Bacillus cereus* spores with either *l*-alanine or *l*-proline as the activator. However, killing was also complete without any added enhancer substance. Although a longer incubation period was required, similar results were obtained using cholesterol oxidase with EPO. In the absence of haloperoxidase, addition of *l*-alanine or *l*-proline did not significantly increase killing activity of cholesterol oxidase. The direct sensitivity of these bacterial endospores to the oxidase-haloperoxidase killing is so great that any effect of *l*-alanine or *l*-proline is hidden in the total kill observed as early as thirty minutes.

The effect of *l*-alanine, *l*-proline, coumarin, and phenethyl alcohol on cholesterol oxidase-haloperoxidase killing of *Aspergillus fumigatus* spores was determined by following the foregoing procedure except that the reaction contained 0.1 unit (i.e., 4 μg) cholesterol oxidase from *Nocardia erythropolis*, 20 pmol (2.8 μg) porcine MPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1899201) or 20 pmol (1.5 μg) porcine EPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1929201), and the indicated quantity of substance to be tested in 50 mM Acetate Buffer containing 100 mEq/L Cl^-, and 1 mEq/L Br^-. The pH was adjusted to ~7 by addition of 50 mM MOPS buffer. The final concentration of cholesterol was 5 mM (5 μmol/ml) in 8.5% ethanol. The final volume was 1 ml. After the indicated period of incubation (37°C), *Aspergillus fumigatus* was plated on Sabouraud's dextrose agar.

The results are expressed in **Table 20** as the colony forming units (CFU) counted. PEA is the acronym for phenethyl alcohol. Of the four potential activator substances tested, *l*-alanine alone increased the kill capacity of cholesterol oxidase with either MPO or EPO. In the absence of haloperoxidase, none of the activators increased the killing activity of cholesterol oxidase.

Table 20

Effects of *l*-Alanine, *l*-Proline, Coumarin, and Phenethyl Alcohol on Cholesterol Oxidase-Haloperoxidase Microbicidal Action Against *Aspergillus fumigatus* Spores:

Treatment:		*A. fumigatus* Survival, CFU (Post Exposure Time in Hours)				
Haloperoxidase	Activator Substance	1 Hr.	1.5 Hr.	2 Hrs.	3 Hrs.	4 Hrs.
None	None	710,000	760,000	530,000	340,000	240,000
None	*l*-alanine, 10 μmol	780,000	720,000	500,000	340,000	340,000
None	*l*-proline, 10 μmol	900,000	540,000	560,000	400,000	200,000
None	coumarin, 1 μmol	320,000	700,000	480,000	240,000	500,000
None	PEA, 5 μmol	380,000	600,000	360,000	440,000	480,000
MPO, 20 pmol	None	520,000	940,000	500,000	410,000	550,000
MPO, 20 pmol	*l*-alanine, 10 μmol	0	0	0	0	0
MPO, 20 pmol	*l*-proline, 10 μmol	860,000	880,000	760,000	700,000	260,000
MPO, 20 pmol	coumarin, 1 μmol	740,000	620,000	500,000	620,000	660,000
MPO, 20 pmol	PEA, 5 μmol	500,000	600,000	420,000	420,000	520,000
EPO, 20 pmol	None	920,000	690,000	840,000	710,000	760,000
EPO, 20 pmol	*l*-alanine, 10 μmol	0	0	0	0	0
EPO, 20 pmol	*l*-proline, 10 μmol	1,100,000	840,000	1,000,000	500,000	520,000
EPO, 20 pmol	coumarin, 1 μmol	760,000	780,000	420,000	340,000	300,000
EPO, 20 pmol	PEA, 5 μmol	780,000	600,000	480,000	460,000	320,000

Incorporation of *l*-alanine into the formulation greatly increases the fungicidal action of oxidase-haloperoxidase against both spores and vegetative forms. *L*-alanine appears to provide the amine nitrogen, CO_2, acetyl-CoA, and reducing equivalents required for germination and growth and as such labilizes the fungi to the action of haloperoxidases. As previously shown with haloperoxidase-glucose oxidase killing (**Table 3**), *l*-proline is not effective as an enhancer substance.

Example 11

Binary Antiseptic Consisting of (1) Cholesterol Oxidase-Haloperoxidase and (2) Cholesterol in Solid Form.

The cholesterol oxidase-haloperoxidase system was constructed as a binary formulation consisting of: (1) a solution containing cholesterol oxidase-haloperoxidase plus the antimicrobial activity enhancing agent *l*-alanine, and (2) cholesterol in the form of a solid wafer. The cholesterol wafers were prepared by dissolving cholesterol in ether, pouring the cholesterol solution on a surface, and allowing the ether to evaporate. The resulting sheet of cholesterol was cut into appropriately sized rectangles of approximately 10 mg.

Cholesterol wafers (approximately 10 mg/wafer) were placed in test tubes containing *Aspergillus fumigatus* spores. Cholesterol oxidase with and without MPO or EPO was added to the tubes to initiate fungicidal action. The system was tested with and without *l*-alanine, and with and without ethanol. Ethanol was added to test its capacity to facilitate cholesterol solubilization. Increased cholesterol solubility would mean increased cholesterol available as substrate for the oxidase. Once initiated, the system was allowed to incubate at ambient temperature (22°C) for time intervals ranging from thirty minutes to four hours. The reaction contained 0.1 units (i.e., 4 µg) cholesterol oxidase from *Nocardia erythropolis*, 10 pmol (1.4 µg) porcine MPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1899201) or 10 pmol (0.7 µg) porcine EPO (ExOxEmis, Inc., San Antonio, Texas, U.S.A., Lot#1929201) in 50 mM Acetate Buffer containing 100 mEq/L Cl^-, and 1 mEq/L Br^-. The pH was adjusted to ~7 by addition of 50 mM MOPS buffer. The substrate cholesterol was present in the form of a solid wafer (approximately 10 mg). Where indicated the reaction contained 10 µmol/ml *l*-alanine and 10% ethanol. The final volume was 1 ml. After the indicated period of incubation (22°C), *Aspergillus fumigatus* was plated on Sabouraud's dextrose agar. The results are expressed as the colony forming units (CFU's) counted. ND indicates that the experiment was not done. The results are presented in **Table 21**.

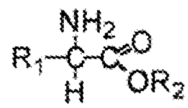
Table 21
Effects of *l*-Alanine on Cholesterol Oxidase-Haloperoxidase Microbicidal Action Against *Aspergillus fumigatus* Spores Using Solid Cholesterol as Substrate With and Without Ethanol:

Treatment:			*A. fumigatus* Survival, CFU (Post Exposure Time in Hours)			
Haloperoxidase	*l*-Alanine	Ethanol	0.5 Hr	1 Hrs.	2 Hrs.	4 Hrs.
None	None	10 %	960,000	1,900,000	960,000	800,000
None	10 μmol	10 %	1,180,000	1,220,000	820,000	158,000
MPO, 10 pmol	None	10 %	920,000	1,540,000	1,120,000	1,020,000
MPO, 10 pmol	10 μmol	10 %	82,000	0	0	0
EPO, 10 p*mol*	None	10 %	1,120,000	3,000,000	3,040,000	940,000
EPO, 10 p*mol*	10 μmol	10 %	0	0	0	0
None	None	None	ND	2,300,000	ND	980,000
None	10 μmol	None	ND	1,980,000	ND	1,900,000
MPO, 10 pmol	None	None	ND	2,380,000	ND	1,860,000
MPO, 10 pmol	10 μmol	None	ND	1,640,000	ND	10,000
EPO, 10 p*mol*	None	None	ND	2,620,000	ND	1,340,000
EPO, 10 p*mol*	10 μmol	None	ND	1,340,000	ND	0

As shown in **Table 21**, in the presence of ethanol plus *l*-alanine, the cholesterol oxidase-MPO formulation effected a tenfold killing of the spores at thirty minutes and complete killing at one, two, and four hours. *L*-alanine was required for effective MPO-dependent killing. In the presence of ethanol and *l*-alanine but absence of haloperoxidase, cholesterol oxidase was mildly effective but only after four hours incubation. The most effective formulation was cholesterol oxidase-EPO with ethanol and *l*-alanine. Complete spore killing was observed for all test intervals.

Inclusion of ethanol greatly increased the effectiveness of all the formulations tested. Sporadical action is faster and more intense in the presence of 10% ethanol. This ethanol effect may result from increased solubilization of the solid cholesterol resulting in increased cholesterol oxidase activity. The results also demonstrate that 10% ethanol has no detrimental effect on the cholesterol oxidase-haloperoxidase function. Inclusion of an adequate quantity of ethanol or other nontoxic solvent in the formulation also assists in maintaining sterility of the system in the absence of cholesterol and improves the shelf-life of the reaction mixture.

1. A method for killing or inhibiting the growth of yeast or sporular microorganisms comprising contacting the microorganisms, in the presence of a peroxide and chloride or bromide, with a haloperoxidase and at least one antimicrobial activity enhancing agent of the formula:



wherein R_1 is hydrogen, an unsubstituted, or hydroxy or amino substituted, straight or branched chain alkyl group having from 1 to 6 carbon atoms, or an unsubstituted, or hydroxy or amino substituted arylalky group having from 7 to 12 carbon atoms, and R_2 is hydrogen or a straight or branched chain alkyl group having from 1 to 6 carbon atoms.

2. The method of Claim 1 wherein the haloperoxidase is selected from the group consisting of myeloperoxidase, eosinophil peroxidase and combinations thereof.

3. The method of Claim 1 wherein the antimicrobial activity enhancing agent is an α-amino acid selected from the group consisting of glycine and the *l*- or *d*-enantiomers of alanine, valine, leucine, isoleucine, serine, threonine, lysine, phenylalanine, tyrosine and the alkyl esters thereof.

4. The method of Claim 2 wherein the haloperoxidase is myeloperoxidase and the halide is chloride or bromide.

5. The method of Claim 2 wherein the haloperoxidase is eosinophil peroxidase and the halide is bromide.

6. The method of Claim 1 wherein the microorganism is contacted with a liquid solution comprising at least 0.01 pmol/ml of haloperoxidase and at least 0.005 μmol/ml of the antimicrobial activity enhancing agent.

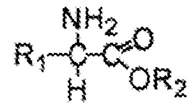
7. The method of Claim 6 wherein the liquid solution comprises from 0.1 pmol/ml to 500 pmol/ml of haloperoxidase and from 0.05 μmol/ml to 50 μmol/ml of the antimicrobial activity enhancing agent.

8. The method of Claim 6 wherein the solution comprises from 0.5 pmol to 50 pmol haloperoxidase per ml of the solution.

Various modifications and adaptations of the methods and compositions of the invention will be apparent from the foregoing to those skilled in the art. Any such modifications and adaptations are intended to be within the scope of the appended claims except insofar as precluded by the prior art.

9. The method of Claim 6 wherein the liquid solution further comprises a peroxide or an agent capable of producing a peroxide.

10. A composition for killing or inhibiting the growth of yeast or sporular microorganisms comprising a haloperoxidase and at least one antimicrobial activity enhancing agent of the formula:



wherein R_1 is hydrogen, an unsubstituted, or hydroxy or amino substituted, straight or branched chain alkyl group having from 1 to 6 carbon atoms, or an unsubstituted, or hydroxy or amino substituted arylalky group having from 7 to 12 carbon atoms, and R_2 is hydrogen or a straight or branched chain alkyl group having from 1 to 6 carbon atoms.

11. The composition of Claim 10 wherein the haloperoxidase is myeloperoxidase or eosinophil peroxidase.

12. The composition of Claim 10 wherein the antimicrobial activity enhancing agent is an α-amino acid selected from the group consisting of glycine and the *l*- or *d*-enantiomers of alanine, valine, leucine, isoleucine, serine, threonine, lysine, phenylalanine, tyrosine and the alkyl esters thereof.

13. The composition of Claim 11 wherein the haloperoxidase is myeloperoxidase.

14. The composition of Claim 13 which comprises at least 0.01 pmol/ml of myeloperoxidase in a liquid carrier.

15. The composition of Claim 14 which comprises from 0.1 pmol/ml to 500 pmol/ml of myeloperoxidase.

16. The composition of Claim 14 which comprises from 0.5 pmol/ml to 50 pmol/ml of myeloperoxidase.

17. The composition of Claim 12 which comprises at least 0.005 μmol/ml of the antimicrobial activity enhancing agent in a liquid carrier.

18. The composition of Claim 17 which comprises from 0.05 µmol/ml to 50 µmol/ml of the antimicrobial activity enhancing agent.

19. The composition of Claim 11 wherein the haloperoxidase is eosinophil peroxidase and the halide is bromide.

20. The composition of Claim 19 which comprises at least 0.01 pmol of eosinophil peroxidase in a liquid carrier.

21. The composition of Claim 19 which comprises from 0.1 pmol to 500 pmol per ml of eosinophil peroxidase in a liquid carrier.

22. The composition of Claim 20 which comprises from 0.5 pmol/ml to 50 pmol/ml of eosinophil peroxidase.

23. The composition of Claim 20 which further comprises from 10 nmol/ml to 10 µmol/ml of bromide.

24. The composition of Claim 10 which further comprises hydrogen peroxide or an oxidase capable of producing a peroxide in the presence of a substrate for the oxidase.

25. The composition of Claim 24 which comprises a peroxide producing oxidase effective to generate from 100 pmol to 50 µmol peroxide per ml per minute when in the presence of a substrate for the oxidase.

26. A disinfecting-sterilizing solution which comprises a composition of Claim 10.

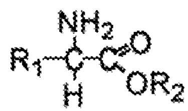
27. The disinfecting-sterilizing solution of Claim 26 which comprises from 0.1 pmol/ml to 500 pmol/ml of myeloperoxidase, eosinophil peroxidase or combinations thereof, and from 0.005 µmol/ml to 50 µmol/ml of an α-amino acid selected from the group consisting of glycine and the *l*- or *d*-enantiomers of alanine, valine, leucine, isoleucine, serine, threonine, lysine, phenylalanine, tyrosine and the alkyl esters thereof, in a liquid carrier.

28. An ophthalmic solution which comprises a composition of Claim 10.

29. The ophthalmic solution of Claim 8 which comprises from 0.1 pmol/ml to 500 pmol/ml of myeloperoxidase, eosinophil peroxidase or combinations thereof, and from 0.005 μmol/ml to 50 μmol/ml of an α-amino acid selected from the group consisting of glycine and the *l*- or *d*-enantiomers of alanine, valine, leucine, isoleucine, serine, threonine, lysine, phenylalanine, tyrosine and the alkyl esters thereof, in a liquid carrier.

AMENDED CLAIMS
[received by the International Bureau on 29 December 1994 (29.12.94);
original claims 1,3-5,9-12,19,24 and 26-29 amended;
remaining claims unchanged (4 pages)]

1. A method for killing or inhibiting the growth of yeast or sporular microorganisms comprising contacting the microorganisms, in the presence of a peroxide and chloride or bromide, with a haloperoxidase and at least one antimicrobial activity enhancing agent of the formula:



wherein R_1 is hydrogen, an unsubstituted, or hydroxy or amino substituted, straight or branched chain alkyl group having from 1 to 6 carbon atoms, and R_2 is hydrogen or a straight or branched chain alkyl group having from 1 to 6 carbon atoms.

2. The method of Claim 1 wherein the haloperoxidase is selected from the group consisting of myeloperoxidase, eosinophil peroxidase and combinations thereof.

3. The method of Claim 1 wherein the antimicrobial activity enhancing agent is an α-amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, lysine, and the alkyl esters of any of the members of the group.

4. The method of Claim 2 wherein the haloperoxidase is myeloperoxidase.

5. The method of Claim 2 wherein the haloperoxidase is eosinophil peroxidase.

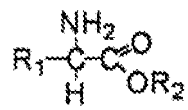
6. The method of Claim 1 wherein the microorganism is contacted with a liquid solution comprising at least 0.01 pmol/ml of haloperoxidase and at least 0.005 μmol/ml of the antimicrobial activity enhancing agent.

7. The method of Claim 6 wherein the liquid solution comprises from 0.1 pmol/ml to 500 pmol/ml of haloperoxidase and from 0.05 μmol/ml to 50 μmol/ml of the antimicrobial activity enhancing agent.

8. The method of Claim 6 wherein the solution comprises from 0.5 pmol to 50 pmol haloperoxidase per ml of the solution.

9. The method of Claim 6 wherein the liquid solution further comprises a peroxide or an agent that produces a peroxide.

10. A composition for killing or inhibiting the growth of yeast or sporular microorganisms comprising a haloperoxidase and at least one antimicrobial activity enhancing agent of the formula:



wherein R_1 is hydrogen, an unsubstituted, or hydroxy or amino substituted, straight or branched chain alkyl group having from 1 to 6 carbon atoms, and R_2 is hydrogen or a straight or branched chain alkyl group having from 1 to 6 carbon atoms.

11. The composition of Claim 10 wherein the haloperoxidase is myeloperoxidase, eosinophil peroxidase and combinations thereof.

12. The composition of Claim 10 wherein the antimicrobial activity enhancing agent is an α-amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, lysine, and the alkyl esters of any of the members of the group.

13. The composition of Claim 11 wherein the haloperoxidase is myeloperoxidase.

14. The composition of Claim 13 which comprises at least 0.01 pmol/ml of myeloperoxidase in a liquid carrier.

15. The composition of Claim 14 which comprises from 0.1 pmol/ml to 500 pmol/ml of myeloperoxidase.

16. The composition of Claim 14 which comprises from 0.5 pmol/ml to 50 pmol/ml of myeloperoxidase.

17. The composition of Claim 12 which comprises at least 0.005 µmol/ml of the antimicrobial activity enhancing agent in a liquid carrier.

18. The composition of Claim 17 which comprises from 0.05 μmol/ml to 50 μmol/ml of the antimicrobial activity enhancing agent.

19. The composition of Claim 11 wherein the haloperoxidase is eosinophil peroxidase.

20. The composition of Claim 19 which comprises at least 0.01 pmol of eosinophil peroxidase in a liquid carrier.

21. The composition of Claim 19 which comprises from 0.1 pmol to 500 pmol per ml of eosinophil peroxidase in a liquid carrier.

22. The composition of Claim 20 which comprises from 0.5 pmol/ml to 50 pmol/ml of eosinophil peroxidase.

23. The composition of Claim 20 which further comprises from 10 nmol/ml to 10 μmol/ml of bromide.

24. The composition of Claim 10 which further comprises hydrogen peroxide or an oxidase that produces a peroxide in the presence of a substrate for the oxidase.

25. The composition of Claim 24 which comprises a peroxide producing oxidase effective to generate from 100 pmol to 50 μmol peroxide per ml per minute when in the presence of a substrate for the oxidase.

26. A disinfecting-sterilizing solution which comprises the composition of Claim 10.

27. A disinfecting-sterilizing solution of Claim 26 which comprises from 0.1 pmol/ml to 500 pmol/ml of myeloperoxidase, eosinophil peroxidase or combinations thereof, and from 0.005 μmol/ml to 50 μmol/ml of an α-amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, lysine, and the alkyl esters of any of the members of the group, in a liquid carrier.

28. An ophthalmic solution which comprises the composition of Claim 10.

29. An ophthalmic solution of Claim 8 which comprises from 0.1 pmol/ml to 500 pmol/ml of myeloperoxidase, eosinophil peroxidase or combinations thereof, and from 0.005 μmol/ml to 50 μmol/ml of an α-amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, lysine, and the alkyl esters of any of the members of the group, in an opthalmically acceptable liquid carrier.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/08608

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/02, 9/08; A61K 38/44; A01N 33/00
US CL :435/189, 192, 264; 424/94.4; 514/579
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/189, 192, 264; 424/94.4; 514/579

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

none

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,473,550 (ROSENBAUM ET AL) 25 September 1984, see entire document.	1-3, 6-18, 20-22, 24-29
Y	US, A, 4,588,586 (KESSLER ET AL) 13 May 1986, see entire document.	1-3, 6-18, 20-22, 24-29
A	US, A, 4,937,072 (KESSLER ET AL) 26 June 1990, see entire document.	1-29

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

31 OCTOBER 1994

Date of mailing of the international search report

16 NOV 1994

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